*SIDE-CHAIN OPTICAL ACTIVITY IN CYSTINE-CONTAINING PROTEINS: CIRCULAR DICHROISM STUDIES**

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There have been several reports of optical activity associated with side-chain chromophores in proteins and polypeptides within the last few years.[1-8] That the sources of optical activity in these cases were side-chain electronic transitions was inferred from the location of actual Cotton effects or irregularities in the optical rotatory dispersion curves in the spectral region 260–300 mμ. In two of the more recent papers[6, 8] circular dichroism measurements were reported. The occurrence and measurements of side-chain optical activity in proteins is important for several reasons. Estimates of helix content by analysis of optical rotatory dispersion curves[9, 10] depend on the absence of Cotton effects at wavelengths longer than about 230 mμ or on ability to subtract such Cotton effects from the background rotation which is due to peptide bond transitions in the 180–225-mμ region. An added complication is that optical activity in the 260–300-mμ region heralds the absence of symmetry of one or more side-chain chromophores and makes it highly probable that Cotton effects due to other transitions in these chromophores will occur at shorter wavelengths where they may go undetected but nonetheless make substantial contributions to optical rotation at longer wavelengths. Finally, because not all residues which absorb light in this spectral region exhibit optical activity, and to the extent that such optical activity is conformation-dependent, the appearance of side-chain Cotton effects is indicative of hindered rotation about the bond(s) connecting the chromophoric group to the asymmetric α-carbon in native proteins. The identification of the responsible chromophores may thus give valuable information about the tertiary structure of proteins and alteration of this structure consequent to interaction with other molecules, change of pH, temperature, and other factors affecting protein structure.

In this paper, we report results of circular dichroism measurements of several proteins in the 250–350-mμ region of the spectrum. Circular dichroism possesses the inherent advantage of presenting discrete bands whose widths are comparable to the associated spectral bands.[11] Thus, while the limbs of a Cotton effect are of different sign on each side of the maximum of the absorption band and extend over hundreds of millimicrons, an ellipticity band has a single sign (which may be positive or negative) and a half band width which may be no greater than 15–20 mμ. A further and substantial advantage of circular dichroism over optical rotatory dispersion in these measurements is that there is no background signal. Thus, a decision as to the wavelength of a band maximum may be made with considerable confidence from the circular dichroism spectrum.

In this paper, we are primarily interested in the optical activity of electronic transitions associated with the disulfide bond at wavelengths longer than 245 mμ. These transitions are only weakly allowed (electrically) in cystine-containing peptides.[12] In proteins, the disulfide spectral bands are difficult to discern be-

cause of the strongly allowed aromatic transitions in this region of the spectrum. At these wavelengths, rotational bands in proteins may arise from tryptophan, tyrosine, and phenylalanine, as well as cystine, and the sorting out of these contributions is no simple task. In this communication, we indicate how the assignment may be made for insulin. Subsequent reports will examine several other proteins which exhibit side-chain optical activity. We have observed ellipticity bands between 250 and 300 mμ, of varying magnitude, sign, and location, in more than a dozen proteins (two of which contain no cystine) and believe that the presence of such optical activity is considerably more widespread among proteins than has formerly been believed.

Experimental.—Circular dichroism measurements were performed on a modified Jouan Dichrographe. The sensitivity is such that a full-scale deflection—250 mm—represents a Δ O.D. of 360×10^{-5}. Average noise levels in the 250–300-mμ region of the spectrum were about 3.0×10^{-5}, and about 7.5×10^{-5} in the 215–240-mμ region. Signal-to-noise ratios at band maxima frequently were as high as 20:1 and are given in the figure legends for several spectra. Expressions for the conversion of these optical density differences to molar ellipticities as well as equations for relating circular dichroism to optical rotatory dispersion are given in an earlier paper.[6]

Crystalline beef insulin was obtained from the British Drug Houses, Ltd. (Batch no. 2189 is from the single lot prepared at the request of the Commission on Proteins, IUPAC.) Hen egg lysozyme, lot no. 14, three times crystallized, and ribonuclease, lot no. 133, five times crystallized, were obtained from Pentex, Inc. Concentrations of the proteins were determined by optical density determinations at the maxima near 280 mμ and from the extinction coefficients cited by Wetlaufer.[12]

Oxidized glutathione was purchased from the California Corp. for Biochemical Research. L-cystine disulfoxide and L-cysteine sulfinic acid were kindly given to us by Dr. Reinhold Benesch. All other chemicals were reagent grade.

Temperatures recorded are those of circulating water leaving the cell compartment. Near 25°C, this temperature did not differ from the bath temperature by more than 0.1°C.

Results.—In Figure 1 are shown the circular dichroism (C.D.) spectra of insulin at various pH values between 8.4 and 11.5 in the wavelength interval 250–310 mμ. These curves were measured less than 10 min after the solutions were prepared. The C.D. spectrum of insulin at pH 2.0 in the same wavelength interval is coincident with the one at pH 8.4. At pH 11.5, the spectrum changes slowly with time, but for the first 30 min after preparation, these changes, if they have already occurred, could not be detected. Figure 2 shows measurements of these same solutions at shorter wavelengths. At about 222 mμ, a negative extremum is encountered in all the curves except for the one corresponding to insulin in 8 *M* urea. This extremum is characteristic of the right-handed α-helix in polypeptides and proteins.[13, 14] (In the random coil form of the polypeptides, an ellipticity band of opposite sign and much-reduced magnitude occurs with a maximum at about 218 mμ.[14, 15]) For a given protein, significant diminution in the intensity of this band concomitant with some treatment may be taken as indicative of lowered helix content and destruction, in some degree, of the native structure. The results in Figure 2 indicate that little or no alteration in helix content occurs on varying pH between 8.4 and 11.5.

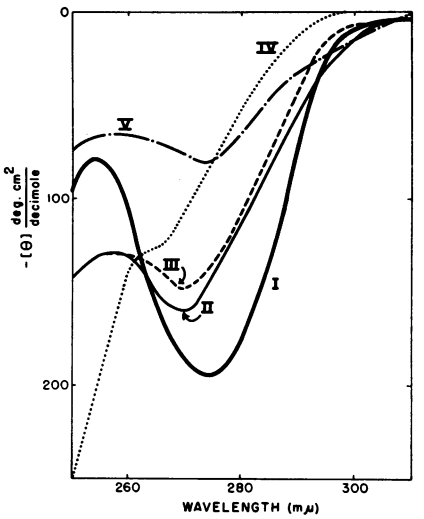

FIG. 1.—Circular dichroism spectra between 250 and 310 mμ of insulin at several pH values and in 8 *M* urea. Curve *I*, pH 8.4–8.6, average of seven spectra; curve *II*, pH 10.55, average of three spectra; curve *III*, pH 10.96, average of three spectra; curve *IV*, pH 11.52, average of five spectra; curve *V*, pH 8.5 in 8 *M* urea. All at 0.1 ionic strength in KCl, KOH solutions. For curve *I*, the signal-to-noise ratio at 273 mμ is 8:1. 25°C.

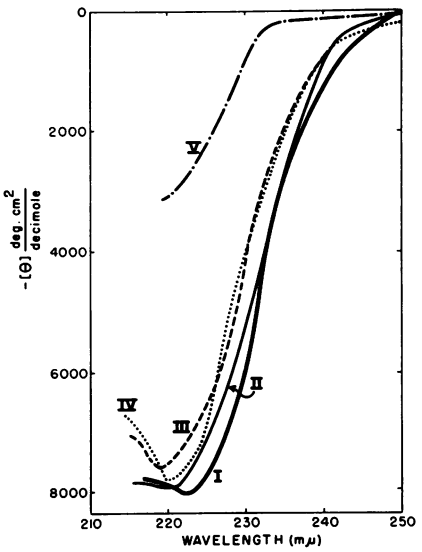

FIG. 2.—Circular dichroism spectra between 215 and 250 mμ of insulin at several pH values and in 8 *M* urea. Curve *I*, pH 8.4–8.6; *II*, pH 10.55; *III*, pH 10.96; *IV*, pH 11.52; *V*, pH 8.5 in 8 *M* urea. For curves *I*, *II*, *III*, and *IV*, the signal-to-noise ratio at 222 mμ is about 20:1. 25°C.

The small changes in position and intensity of this band are almost within experimental error and are readily explained without need to invoke altered secondary structure (see below). This sensible constancy in the far ultraviolet may be contrasted with the notable changes, particularly at pH 11.5, at the longer wavelengths, seen in Figure 1. The maximum in the absorption spectrum of insulin shifts to 294 mμ at pH just below 11,[16] at this ionic strength. In insulin, ionization of tyrosine residues is noteworthy for reversibility and for the fact that all of the tyrosine residues appear to be in prototropic equlibrium in the native protein.[16, 17] It is thus significant that the ellipticity band of Figure 1 shows no displacement to longer wavelength with increasing pH. At pH 11.5, the band(s) is qualitatively different but occurs at *shorter* rather than longer wavelength. A change in the chromophore responsible for this band must have occurred, but it is improbable that this band is associated with tyrosine residues.

Insulin maintained at pH 11.5, 25°C, for an hour, then returned to pH 8.4, exhibits the same C.D. spectrum as material at pH 8.4 which has not been exposed to more alkaline pH. The C.D. spectrum at pH 11.5 does change with time, albeit slowly. After 20 hr (Fig. 3), the long-wavelength ellipticity band has disappeared. Restoration of this solution to pH 8.4 restores the band, although the intensity is now about one half the value observed in solutions at this pH which have not been exposed to alkali. This result may be contrasted with the C.D. spectra at shorter wavelengths (Fig. 4). Here, 20-hr exposure to pH 11.5 drastically alters the peptide ellipticity bands (helix content) but restoration to pH 8.4 after

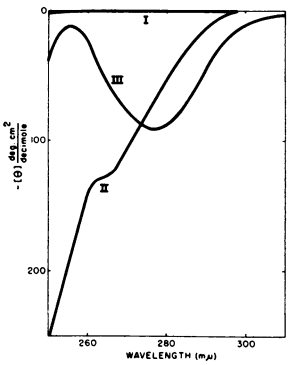

FIG. 3.—Effect of alkali on C.D. spectra of insulin at 250–310 mμ. Curve *I*, solution maintained at pH 11.5 for 20 hr; curve *II*, for comparison, solution freshly prepared at pH 11.5; curve *III*, pH 8.4 after 20 hr at pH 11.5. 25°C.

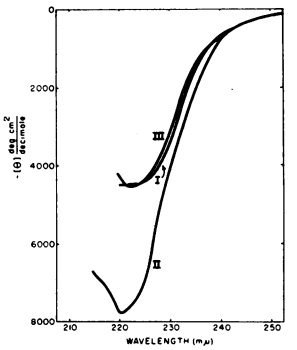

FIG. 4.—Effect of alkali on C.D. spectra of insulin at 215–250 mμ. Curve *I*, solution maintained at pH 11.5 for 20 hr; curve *II*, for comparison, solution freshly prepared at pH 11.5; curve *III*, pH 8.4 after 20 hr at pH 11.5. 25°C.

20 hr brings about no increase of intensity at 222 mμ and, we infer, no restoration of helix content. Thus the structural elements responsible for ordering those side-chain residues which are optically active do not directly depend on, and may be unrelated to, helix content. The addition of mercaptoethanol (or cysteine) to insulin at pH 8.5 leads to the same result as long exposure to alkali. Depending on the concentration of thiol (10^{-3} to 10^{-2} *M*), the long-wavelength ellipticity band can be abolished within minutes after adding thiol compounds and the short-wavelength band achieves the same value as observed at pH 11.5 after 20 hr. It is interesting, in this connection, that 8 *M* urea clearly leads to different structural changes than are brought about by long exposure to alkali or addition of thiols. In 8 *M* urea (Figs. 1 and 2) the intensity of the ellipticity at 222 mμ is lower than observed for the alkaline solution, but a long-wavelength ellipticity band is present in contrast to the solution held at pH 11.5 for 20 hr.

Figure 5 shows the C.D. spectra of L-cystine, oxidized glutathione, L-cystine disulfoxide, and cysteine sulfinic acid. The ellipticities are expressed per mole (see also Table 1). In L-cystine, the position of the long-wavelength negative xtremum corresponds approximately to the position of the shoulder at 250 mμ

TABLE 1

MOLECULAR ELLIPTICITIES AT BAND MAXIMA BETWEEN 240 AND 300 mμ

Compound	pH	$[\Theta]_{MRW}$	$[\Theta]_{cyst.}$	$[\Theta]_{tyr.}$	$[\Theta]_{try.}$	λ_{max}(mμ)
Cystine	INH_2SO_4	...	−1760	...	...	248
Cystine	H_2O	...	−3750	...	...	255
Glutathione(ox)	H_2O	−350	−1390	...	...	260–261
Insulin	2.0, 8.5	−195	−1815	−1715	...	273–275
RNAase	7.0	−215	−3625	−3030	...	273
	7.0	+110	+1855	+1550	...	242
Lysozyme	2.0 and 7.0	+130	+1460	+1990	+1326	283
		−120	−1350	−1840	−1225	252–258

$[\Theta]_{MRW}$ is calculated on the basis of total molarity of peptide bonds. $[\Theta]_{cyst.}$, $[\Theta]_{tyr.}$, and $[\Theta]_{try.}$ are calculated on the basis of molarity of cystine, tyrosine, and tryptophan *residues*, respectively, in the proteins and in oxidized glutathione.

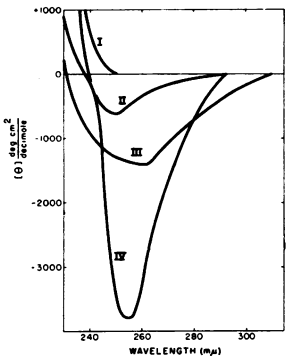

FIG. 5.—Circular dichroism spectra of cystine and related compounds between 230 and 310 mμ. Curve *I*, cysteine sulfinic acid in water; curve *II*, cystine disulfoxide in 0.1 *N* H_2SO_4; curve *III*, oxidized glutathione in H_2O; curve *IV*, cystine in H_2O (0.01%). For cystine, at 255 mμ, signal-to-noise ratio is slightly greater than 25:1. 25°C.

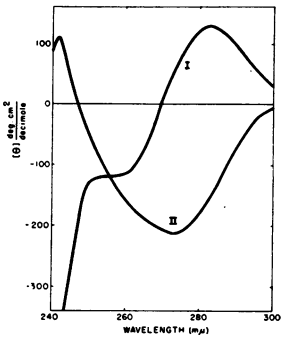

FIG. 6.—Circular dichroism spectra of ribonuclease and lysozyme between 240 and 300 mμ. Curve *I*, lysozyme at pH 7.0 in 0.1 ionic strength phosphate buffer. Curve *II*, ribonuclease at pH 7.0 in 0.1 ionic strength phosphate buffer. At 273 mμ, signal-to-noise ratio for curve *II* is 22:1. At 282 mμ, signal-to-noise ratio for curve *I* is slightly less than 6:1. 25°C.

in the absorption spectrum of this amino acid.[18] The position of the band in oxidized glutathione is displaced toward longer wavelengths by about 5 mμ. Other derivatives of cystine which have either the amino or carboxyl, or both, incorporated into peptide bonds show displacement of the ellipticity band to longer wavelengths.[19] The disulfoxide shows a considerable reduction in magnitude of the long-wavelength band. It is shown here because of its possible interest as an intermediate in the route of oxidation to the cysteic acids.

To indicate the position and magnitude of long-wavelength ellipticity bands in two other proteins containing cystine residues, we show, in Figure 6, the circular dichroism spectra of ribonuclease and lysozyme between 230 and 300 mμ. Each protein exhibits two ellipticity bands of opposite sign in this spectral interval.

Discussion.—Because of restricted rotation about the —S—S— bond in a dialkyl disulfide, two mirror image rotamers presumably exist in solution. The dihedral angle in such a disulfide is approximately 90° and the barrier to rotation about the disulfide bond is at least 10 kcal.[18, 20] Each rotamer has a distinct screw sense and superimposition is not possible. In L-cystine, the presence of optical activity in a transition known to be associated with sulfur electrons makes it probable that one screw sense is preferred. In a native protein, it seems likely that there will be restricted rotation about any given disulfide bond and this would lead to optical activity associated with transitions localized at the —S—S— bond. That the dihedral angle in L-cystine is near 90° may be inferred from the position of the absorption band near 250 mμ. In a cyclic alkyl disulfide, such as 1,2-dithiolane, the dihedral angle is much reduced—to about 27°—and the absorption band occurs at about 330 mμ.[20] Theoretical reasons for this shift have been given.[20] If, as the results of this paper suggest, incorporation in peptide structure elevates the wavelengths of the absorption maximum by 10 to 20 mμ, this may suggest a dihedral

angle somewhat less than 90°. However, two distinct screw senses in cystine residues are still to be expected, with opposite sign ellipticity associated with disulfide transitions. In such a chromophore, the nearby asymmetric centers determine the dissymmetry of the chromophore and will influence the magnitude of the optical activity, but the sign of the rotation and ellipticity is determined by the screw sense.[21]

These remarks are intended to underline the author's belief that cystine residues in native proteins should, *in general*, give rise to ellipticity bands in the 250–270-mμ region. However, there is no structural information about proteins to indicate that the sign of ellipticity should be the same for all cystine residues. Whether a given disulfide bond adopts one screw sense or another probably depends on the size of the loop, the steric requirements of adjacent residues, electrostatic interactions, etc. One result of this may be cancellation of optical activity due to contributions of opposite sign.

In view of the fact that tyrosine ionization in insulin leads to no red shift of the long-wavelength ellipticity band and because no tryptophan is present in insulin, we conclude that this band is predominantly due to a disulfide transition. That the band is displaced to shorter wavelength with increasing pH would appear to indicate that increasing net negative charge on the molecule, or certain proton dissociations, in particular, lead to increase in the dihedral angle of one or more of the cystine residues. The curve obtained with freshly prepared insulin at pH 11.5 may indeed result from the presence of two negative ellipticity bands of slightly different wavelength for the maxima, but both due to disulfide transitions in different cystine residues. Notably, this occurs without significant alteration of helix content in insulin. In urea, the helix content is markedly diminished. If all three disulfides in the monomer unit (mol. wt. 5700) are optically active, and contribute approximately equally to the observed ellipticity of the native protein, then the reversal of disulfide screw sense in one of these when 8 *M* urea is added would give the observed result, since the intensity at the band maximum is reduced by about two thirds.

The most interesting observations in insulin, in this connection, are those involving exposure to pH 11.5 for 20 hr and reversal to pH 8.4. At pH 11.5, the presence of small amounts of thiol due to hydrolytic cleavage of cystine residues[22] would lead to reduction and disulfide interchange.[23] Extensive cancellation of opposite sign rotatory strengths would then be expected. It is remarkable, however, that restoration to pH 8.4 restores the ellipticity band to one half its former size without return to native secondary structure.

In this discussion, phenylalanine residues have been omitted from consideration as a source of optical activity largely because we have observed that the amino acid shows very little optical activity associated with long-wavelength ring transitions, and others have reported the same.[24] Our examination of phenylalanine residues in various peptides is in progress, but it is unlikely that phenylalanine residues could make any but a small contribution to the ellipticity at long wavelengths observed in insulin.

In ribonuclease, the positive band is at too short a wavelength to be a disulfide transition and is compatible, in position, with an optically active tyrosine band.[6] The longest-wavelength negative ellipticity band may be due to tyrosine, cystine, or

both. In lysozyme, the shorter-wavelength band (which appears as a step or shoulder) is probably due to a disulfide transition and the longer-wavelength, positive band may be contributed to by tyrosine, tryptophan, and cystine. That the longest-wavelength ellipticity bands in ribonuclease and lysozyme differ in sign does not seem to us incompatible with possible contribution to each by disulfide transitions.

In ribonuclease and in lysozyme some of the tyrosine residues (and possibly, in lysozyme, tryptophan) have been implicated as being nonreactive in the native proteins.[25, 26] In these cases, systematic examination of the effects of pH, temperature reduction of disulfide bonds, and chemical modification of the reactive tyrosines may lead to an ability to distinguish the contributions to the observed ellipticity from different chromophores. These investigations are under way in our laboratory and will be reported in due course.

Finally, we may note that all the model compounds containing disulfide bonds show some ellipticity in the spectral regions associated with peptide transitions. Coleman and Blout[27] have carried out an extensive investigation of the far UV optical activity associated with the disulfide bond in cystine and many of its derivatives. Small variations in such short-wavelength bands probably account for the differences in ellipticity in the 220-mμ region noted in insulin at different pH's.

Summary.—Ellipticity bands in the circular dichroism spectra of insulin, ribonuclease, and lysozyme occur at wavelengths longer than 240 mμ, and these are due to optically active transitions in the side chains (as distinct from peptide bond transitions at shorter wavelengths). In insulin, variation of pH from 8.4 to 11.5 leads to changes in the circular dichroism spectra which are incompatible with assignment of the bands to tyrosine transitions. The most probable source of optical activity in insulin at these wavelengths is the disulfide chromophore. Circular dichroism spectra of cystine, oxidized glutathione, and cystine disulfoxide exhibit ellipticity bands of substantial magnitude at wavelengths corresponding to the (weakly allowed) absorption bands in these compounds. The effects of long exposure to alkali, and of addition of mercaptoethanol and urea on these ellipticity bands in insulin are presented and discussed in terms of possible alteration of configuration about the disulfide bond in cystine residues. The origin of the bands in lysozyme and ribonuclease is discussed.

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*FACTORS AFFECTING THE REVERSIBLE DISSOCIATION OF DEHYDROGENASES**

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A number of multichain enzymes have been reversibly inactivated by urea, guanidine, or low pH, including aldolase,[1] fumarase,[2] alkaline phosphatase,[3] and α-glycerophosphate dehydrogenase.[4] In some cases, the enzymes have been shown to dissociate into subunits. Epstein *et al.* have recently shown that under appropriate conditions, rabbit muscle lactic dehydrogenase (LDH) can be reactivated after denaturation by 10.5 *M* urea or 5 *M* guanidine, thus demonstrating thermodynamic control of formation of the native conformation of a multichain protein that contains no disulfide bonds.[5]

Malic dehydrogenase (MDH) and LDH are similar in several ways. Both enzymes exist in multiple forms,[6, 7] and bind more than 1 mole of coenzyme per mole of enzyme.[8, 9] Neither contains disulfide bridges and both are readily inactivated by guanidine or urea; such treatment results in complete exposure of sulfhydryl groups to HMB.[10] By several criteria both enzymes have been shown to contain subunits.[6, 11, 12]